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# DIFFERENCES IN POLLINATION SYNDROMES AND THE FREQUENCY OF AUTONOMOUS DELAYED SELFING BETWEEN CO-FLOWERING *HIBISCUS APONEURUS* (SPRAGUE AND HUTCH) AND *H. FLAVIFOLIUS* (ULBR) FROM KENYA

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**Abstract**—Delayed autonomous selfing offers a mechanism for seed production when pollination levels are low or unpredictable. At Mpala Research Centre (MRC) in Kenya, we examined the relationships between floral attraction, insect visitation, and delayed autonomous selfing through backwards stylar curvature in the co-flowering *Hibiscus aponeurus* and *H. flavifolius*. Despite producing similar pollen and nectar rewards, visitation rates and the composition of floral visitor guilds varied significantly between these species. Across four years of observations, floral visitation in *H. flavifolius* was dominated by bees, and in *H. aponeurus* by a mixture of bees, butterflies and beetles. Visitation rates to *H. flavifolius* flowers (range 0.17 - 2.1 visits flr<sup>-1</sup>hr<sup>-1</sup>) were two times greater than to *H. aponeurus* flowers (range 0 - 2.7 visits flr<sup>-1</sup>hr<sup>-1</sup>), which resulted in significantly higher pollen deposition and removal rates in *H. flavifolius* than in *H. aponeurus*. Field crosses demonstrated little pollen limitation in either species. In open-pollinated flowers, *H. aponeurus* displayed significantly greater stylar curvature and apparent self-pollination than did *H. flavifolius*. Floral attributes in *H. aponeurus*, such as a smaller corolla size and a downwards orientation of the stylar column, also suggest that delayed selfing is a more important mechanism of reproductive assurance in this species than in *H. flavifolius*. Determining whether these differences in insect visitation and stylar curvature are characteristic for these species or are unique to MRC will require comparison with populations located in other parts of the ranges, genetic tests of selfing rates, and chemical analyses of nectar, pollen, and floral volatiles.

**Keywords:** Delayed autonomous selfing, *Hibiscus*, pollination, stylar curvature, Mpala Research Centre

## INTRODUCTION

Human activities that destroy natural habitats and fragment populations are reducing the size of pollinator populations and potentially decreasing the reproductive success of many plant species (Aguilar et al. 2006; Eckert et al. 2010; Thomann et al. 2013; Vanbergen 2013; Somme et al. 2012). Habitat fragmentation can increase reliance on self-pollination (e.g. Brys & Jacquemyn 2012) and over time potentially can result in inbreeding depression, reduced genetic variability, and increased risk of local extinction due to the loss of adaptive potential (Stebbins 1957; Goodwillie et al. 2005; Eckert et al. 2010). As reviewed by Wright et al. (2013), however, selfing also has potential benefits, including

a 50 per cent transmission advantage over outcrossing. Further, selfing can sometimes provide reproductive assurance when potential mates are present at low densities, such as during colonization events (e.g. Pannell & Barrett 1998; Rambuda & Johnson 2004), or when pollination rates are low or unpredictable, such as in the decline or absence of suitable pollinators (Zhang et al. 2014).

From a functional standpoint, selfing can be mediated by pollinators (geitonogamy and facilitated autogamy) or it can be autonomous and occur spontaneously within flowers without the aid of a pollinator (Lloyd & Schoen 1992). Of the three modes of autonomous selfing described by Lloyd & Schoen (1992), delayed autonomous selfing is favoured under the widest range of ecological conditions (Morgan & Wilson 2005; Morgan et al. 2005), because it does not occur until opportunities for outcrossing have passed, thereby eliminating seed and pollen discounting. The frequency and importance of autonomous selfing in plant populations show considerable

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variation among individuals (Kalisz et al. 1999), among populations (Klips & Snow 1997), and among closely related species (Brys & Jacquemyn 2011), most likely because selfing rates are strongly influenced by local biotic and abiotic conditions (Kalisz et al. 2004; Qu et al. 2007; Ruan et al. 2009; Vaughton & Ramsey 2010; Jorgensen & Arathi 2013).

Delayed autonomous selfing occurs in several genera of the Malvaceae (Ruan et al. 2010, 2011), but detailed exploration of its ecological importance has been limited to *Hibiscus laevis* (Klips & Snow 1997), *Hibiscus trionum* (Ramsey et al. 2003; Seed et al. 2006), and *Kosteletzkya virginica* (Ruan et al. 2005, 2008a, 2008b, 2009). Most Malvaceae capable of delayed selfing have flowers with styles that are surrounded by and extend beyond monadelphous stamens. In these species, the styles curve out and backwards as flowers age until the stigmas contact the pollen located in the upper anthers (see images in Ruan et al. 2010; Kumar et al. 2014). Studies in *Hibiscus* (e.g. Klips & Snow 1997) suggest that this backward bending can be stopped by prior pollination, although the number of pollen grains required to halt curvature and the physiological mechanism behind that interaction are not well understood (Buttrose et al. 1977; Klips & Snow 1997; Seed et al. 2006; Ruan et al. 2008a).

Here, we report results of a multiple-year study examining the pollination biology, breeding system, and mechanisms for reproductive assurance in *Hibiscus aponeurus* (Sprague & Hutch.) and *Hibiscus flavifolius* (Ulbr.). These species of semiarid Africa are morphologically and ecologically similar, and they co-flower following seasonal rains at Mpala Research Centre (MRC) in Laikipia, Kenya, where we conducted our study. Our initial observations revealed that these species produce abundant pollen and nectar rewards and attract a broad array of insect visitors, but visitation frequency to *H. aponeurus* appeared to be lower than to *H. flavifolius*. Further, at the end of the day when flowers were closing, both species were capable of autonomous selfing through stylar curvature, but this action appeared to be more common in *H. aponeurus*. To examine the links between floral attraction, insect visitation, fruit and seed production, and delayed selfing in these species, we performed a series of field observations and greenhouse manipulations between 2004 and 2013. Specifically, we asked: 1) How do *H. aponeurus* and *H. flavifolius* differ in floral display and in the rewards offered to potential pollinators? 2) Are the insect visitor assemblages of these *Hibiscus* species similar and consistent across years? 3) How do insect visitation rates, levels of pollen deposition, and levels of pollen removal compare between species? 4) Is fruit set and seed production limited by low rates of pollination? 5) How common and effective is delayed selfing as a mechanism of reproductive assurance?

Based on our preliminary observations, we hypothesized that *H. aponeurus* at MRC will more heavily rely on delayed selfing as a mechanism to ensure pollination, and we predicted that for open-pollinated flowers, stylar curvature would be significantly greater in *H. aponeurus* than in *H. flavifolius*. Further, because pollination in other malvaceous species can prevent the curving of stigmas, we predicted that manually pollinated flowers of both species would exhibit less stylar

curvature than would unpollinated, intact flowers allowed to perform autonomous selfing at the end of the day.

## MATERIALS AND METHODS

### Study site and species

Our fieldwork was conducted at Mpala Research Centre (MRC) in Central Kenya (37°52'E, 0°17'N) from 2004–2008 and in 2013. The vegetation at MRC is predominantly savannah woodland, with alternating areas dominated by grasses and forbs (see Baldock et al. 2011; Ruiz-Guajardo 2008, for a more detailed description of vegetation). Throughout MRC, *Hibiscus aponeurus* is less abundant and more patchily distributed than *H. flavifolius*. We concentrated our efforts in two approximately 0.5 Ha plots where both species commonly coflowered in close proximity to one another. For some variables, additional data were collected in 2008 from plants in a greenhouse located at Indiana University South Bend, USA.

Both *Hibiscus* species are erect perennials found in wooded grasslands, but differ subtly in growth habit, microhabitat, flower shape, and more distinctly in flower colour (Agnew & Agnew 1994). *Hibiscus aponeurus* grows mostly underneath and scandently up through acacia trees. In contrast, *H. flavifolius* has sturdier stems, is a better colonizer of open areas grazed by cattle and native herbivores, and is therefore often found growing in grassy glades away from trees. Flowers of both species are solitary in the leaf axils; they open before 0800 hrs, close before 1800 hrs, and do not reopen thereafter. The petals in both species open to produce a nearly flat landing surface for insects that completely exposes the stylar column with its monadelphous stamens (Fig. 1A). Both species produce abundant bright orange pollen, but petals in *H. aponeurus* are bright crimson, while in *H. flavifolius* are pure white. As is typical in *Hibiscus* (Pfeil et al. 2002), the stylar columns of both species have five branches, each of which ends in a capitate stigma. Ovaries of both species contain approximately 30 ovules.

### Floral display

We measured corolla diameters, length of stylar column, and the angle of the stylar column with respect to the nearly vertical plane of the corolla for 22 *H. aponeurus* flowers and 35 *H. flavifolius* flowers. A measurement of 90° indicated that the stylar column was orthogonal to the plane of the corolla, whereas angles greater than 90° indicated that it pointed downwards. To ensure that flowers were fully opened, we conducted all floral measurements between 0830 – 1100 hrs. We compared floral measurements between species using t-tests. All statistical analyses were conducted in JMP v.8.0.1 software (SAS Institute Inc., Cary, North Carolina, USA), and all means are shown  $\pm$  1 S.E.

### Floral abundance and floral rewards

Higher floral abundance often increases visitation rates and may affect pollen transfer, because pollinators do not have to search long before finding the next flower (Elliot & Irwin 2009; Scriven et al. 2013). To estimate seasonal variation in floral abundances, we conducted a total of 18 single-day

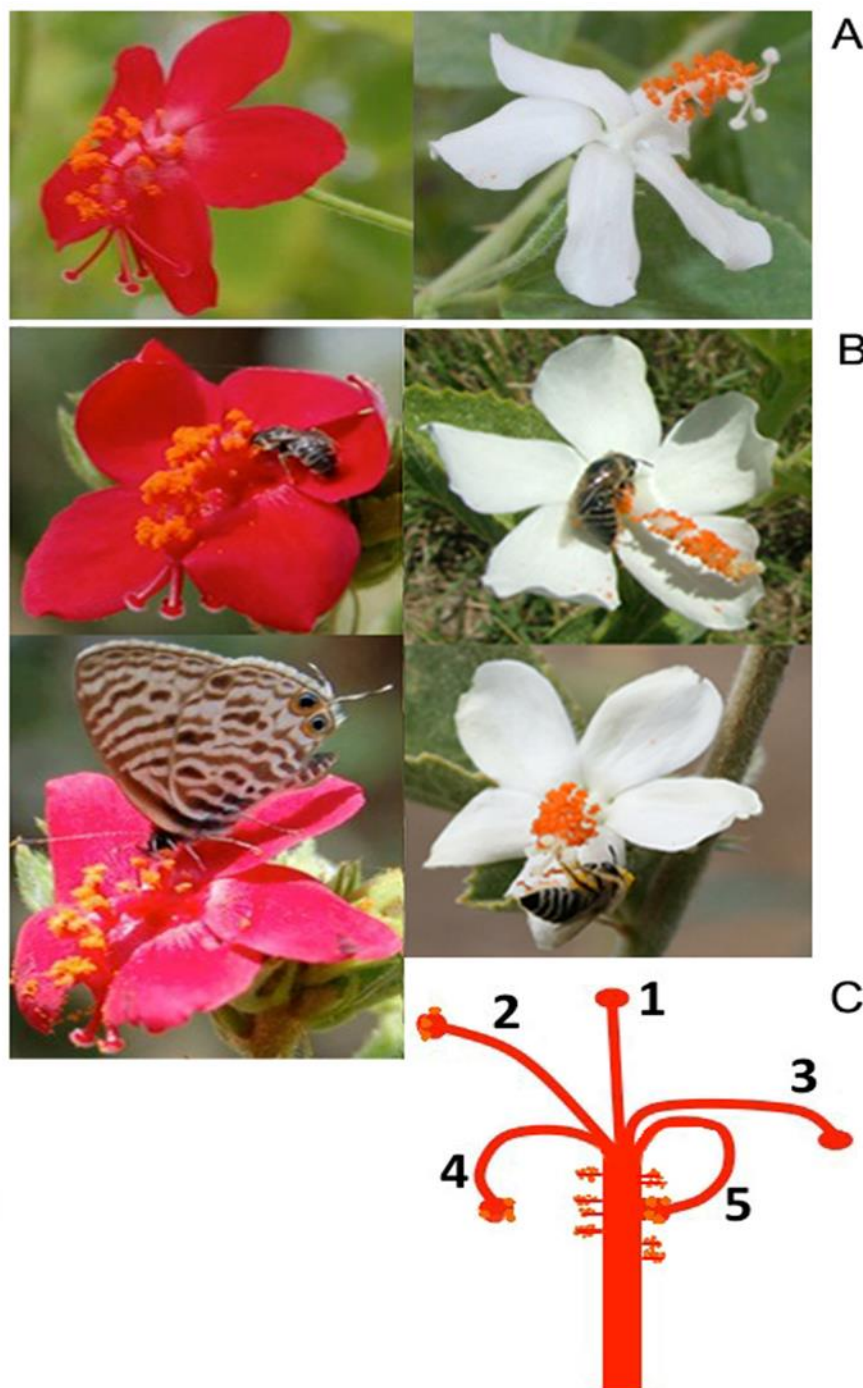


FIGURE 1. Floral morphology, examples of insect visitation behaviour, and stigma behaviour for *Hibiscus aponeurus*, and *Hibiscus flavifolius*. A) Differences between *H. aponeurus* (left) and *H. flavifolius* (right) in staminal tube and stylar orientation relative to the plane of fully opened petals. Note also the somewhat inaccessible location of *H. aponeurus* stigmas. B) Examples of backward facing visitation to *H. aponeurus* by a bee (top left) and a butterfly (bottom left). A contrasting illustration of a backward facing visitation by a bee to an *H. flavifolius* flower (top right), and a forward facing visitation where the bee directly lands on the stigma (bottom right). C) Scoring scheme for stylar curvature showing monadelphous stamens with orange pollen grains, and red styles and stigmas.

surveys between 2004 and 2006. In 2004, we counted the number of open *H. aponeurus* and *H. flavifolius* flowers on one day during each of three weeks in May, one day during each of two weeks in June, July, and August, and on one day in October. Additional single-day counts were made 2005 (March, April, May, October) and 2006 (May, June, July, October).

To examine daily variation in standing crops, we sampled nectar and pollen from unbagged flowers in 2005 and 2006 once each hour in the following 2-hour intervals: 0800 - 0900, 1200 - 1300, and 1600 - 1700 hrs. At each collection,

we sampled 4 - 12 flowers / species from plants not included in the patch used for observations of insect visitation. To measure nectar standing crop volume ( $\mu\text{L}$  / flower), we used glass microcapillaries of known total volumes (Camlab, UK) to probe at the base of the corolla, carefully avoiding to clog the tubes (see Stone et al. 1998). We measured sucrose concentration (% sucrose g / 100 g solution) directly with hand held pocket refractometers modified by the makers (Bellingham & Stanley, UK) for volumes down to 0.15  $\mu\text{L}$ . Sucrose concentrations were later converted into g sucrose / L of nectar using values from tables published in Kearns (1993). For further details about sampling methods, see Stone et al. (1996, 1998) and Raine et al. (2007). The percentage

of pollen remaining in anthers was scored on a scale of 0 - 4 (0 = < 1%; 1 = 1 - 25%; 2 = 26 - 50%; 3 = 51 - 75%; 4 = 76 - 100%). To minimize scoring bias, we spent multiple days calibrating pollen estimates. Each crew member scored several flowers throughout the day, using a fully dehiscent, unvisited flower as the basis for a pollen score of 4 and an emptied flower as the basis for a score of 0. We performed these exercises until sampling was consistent by each crew member. We compared between-species differences in pollen, nectar volume, and sugar standing crops for each time interval and year of observation using nested analysis of variance (ANOVA). We fitted day of observation as a random variable nested within species. Because sampling floral resources often requires the partial destruction of corollas, during 2008 when flowering densities for both species were much lower, we did not sample nectar standing crop and only scored percentage of pollen remaining on the anthers at the end of the day (1630 - 1730 hrs) for the flowers that had been observed for insect visitation. We used nested ANOVA to compare end-of-day pollen scores between the two species.

### *Insect visitation*

Based on the strong daily structuring of floral visitation and high seasonality among plant-visitor interactions reported by Baldock et al. (2011) for our study area, we conducted floral observations that spanned multiple daily time intervals and seasons. In 2004, each species was observed for 20 minutes during each of four time intervals (0600 - 0900, 0900 - 1200, 1200 - 1500, and 1500 - 1800 hrs) on two different days within a two-week period (see Baldock et al. 2011). To obtain an even more detailed representation of the visitation patterns of these plant species, in May and June of 2005, 2006, and 2008, flowers were observed for 40 min each hour from anthesis to closure (0800 - 1800 hrs). Observational patches in 2004, 2005 and 2006 varied from 8 - 15 flowers / species, but in 2008 due to lower flower abundances, patches were smaller (mean of 4 flowers / patch in *H. aponeurus* and 8 flowers / patch in *H. flavifolius*). A visit was recorded whenever an insect landed anywhere on the corolla, or approached the flower and contacted anthers or stigmas. For each *Hibiscus* species, we monitored insect visitation for at least 2 days in 2004 and 2005, 5 days in 2006, and 9 days in 2008. We noticed that many visitors landed on the corolla in an orientation that did not allow contact with the stigmas. Thus, in 2008 we recorded the orientation of the insect on the flower as either frontward facing (body of insect could contact anthers and possibly also stigmas) or backward facing (head near floral tube to allow probing for nectar, but body oriented at some angle away from the distal end of the style, so that contact with stigmas was not possible and even contact with anthers was minimized; see Fig. 1B). Our "backward" category includes approach angles that are similar to, but more extreme than, side-working as understood for apples and similar flowers (e.g. Delaplane et al. 2013).

We grouped insect visitors by order (Hymenoptera, Lepidoptera, Coleoptera, Diptera, and Hemiptera) and for each year independently (2004 - 2008), calculating the proportion of visits accounted by each order and the hourly visitation rates on each day of observation (number of visits  $\text{fl}^{-1}\text{hr}^{-1}$ ). To compare the composition of insect visitor

assemblages between *Hibiscus* species within years, and the proportions of frontward *versus* backward facing visits recorded in 2008, we used chi-square tests or Fisher's exact tests if sample sizes were small. We used t-tests for comparisons of visitation rates between species for each year independently.

### *Tests for apomixis, pollen limitation, autogamy, and reproductive assurance*

We performed floral manipulations in wild populations at MRC during 2005 - 2007 and 2013, and in the greenhouse at Indiana University South Bend in 2008 (see Supplemental Information Appendix I (A) for seed germination and (B) for growing conditions). Rates of fruit set (percentage of total flowers pollinated that developed a fruit) and seed production (number of seeds per mature fruit) were examined for five treatments, although not all treatments were repeated across all years of observation: a) apomixis (seed production without fertilization), b) manual outcrossing (pollen transferred manually from one plant to another), c) manual selfing (pollen transferred manually from the anthers to the stigmas of the same flower), d) autonomous selfing (seeds produced in bagged flowers through delayed selfing), and e) open pollination (seeds produced either through pollen transferred between plants by wild pollinators, through facilitated selfing, or through delayed autonomous selfing). In both species, flower production per plant was low (usually < 3 flowers per day), and flowers were open for a single day only. For the autonomous selfing treatment, we covered flowers with small bags made from fine mosquito net and threaded with cotton cords, which were used to close the sacks tightly and to tie them to the stems. Although great care was exercised to avoid contacting sexual organs, it is possible that some stigmas were accidentally pollinated during bagging or later in the day if wind movement knocked the bags against the stylar column. All treatments were completed shortly after flowers opened in the morning, and bags were removed at the end of the day after the flowers had closed. For all manual pollinations, we used small brushes to collect pollen and gently dab it onto stigmas until all five lobes were completely covered. In 2007, we eliminated facilitated autogamy as a potential mode of pollination by emasculating open-pollinated and manually pollinated flowers.

Regular monitoring of fruit development in the field and in the greenhouse indicated that if pollination was not successful and fruit set was not going to occur, then the ovary did not begin to swell, and flower abscission occurred within a week following treatment. Therefore, we considered fertilization to have occurred successfully, and fruit to have been set, if we recorded the presence of a swelling fruit at least one week after pollination. Although this interpretation of fruit set is not as accurate as following all fruits to dehiscence, it was a necessary aspect of the fieldwork, because complete fruit maturation can take several weeks and during that time many fruits, and sometimes whole plants, were eaten by mammals. For this reason also, sample sizes (number of flowers per treatment) for seed set counts were lower than for the level of fruit set. As a consequence of herbivory and low flower production per plant, statistical analyses for field experiments did not attempt to account for variation among

maternal plants. To assess differences in fruit set among treatments, depending on sample sizes, we used chi-square or Fisher's exact tests, and for seed numbers, we used ANOVA with *post hoc* Tukey-Kramer comparisons.

Treatment comparisons for estimating various pollination parameters followed Eckert et al. (2010). First, we verified that fruits could not be produced by apomixis by either removing all stigma lobes from mature undeveloped flowers (MRC in 2005 and greenhouse in 2008) or brushing away all pollen from anthers and bagging the flowers to exclude pollinators (MRC in 2007). Second, we tested for pollen limitation by comparing levels of fruit and seed production between open-pollinated and manually pollinated flowers (both outcrossed and selfed to help account for variation in pollen quality; see Thomson 2001; Aizen & Harder 2007). Third, we tested for autofertility (the proportion of maximum seed production that can potentially be achieved through autonomous selfing) in the field by comparing fruit and seed production between autonomously selfed flowers with manually outcrossed and manually selfed flowers. We performed an equivalent test for autofertility in the greenhouse, where we eliminated the potential effect of facilitated autogamy by comparing un-manipulated flowers with manually outcrossed and manually selfed flowers. The greenhouse was free of pollinating insects, so delayed selfing was the only possible means of pollination for unmanipulated flowers. Fourth, we conducted a small field test of reproductive assurance in 2007 by comparing fruit set between open-pollinated intact flowers and open-pollinated emasculated flowers.

#### *Use of stylar curvature for delayed selfing*

To examine whether the frequency of stylar curvature for delayed selfing differed between species, we surveyed open-pollinated late afternoon flowers at MRC in 2005, 2006, 2007, and 2008. We scored the degree of stylar curvature using a scale of 1 - 5, allocating an independent score to each of the five stylar branches of the flowers and then taking the average (see Fig. 1C). Our scores represent: (1) upright with little or no curvature, (2) outwards curvature ( $\sim 45^\circ$ ) showing clearly that stigma lobes were spreading apart, (3) curvature to a position close to, but not exceeding  $90^\circ$  to the long axis of stylar column, (4) curvature  $> 90^\circ$  but the stigma not touching the anthers, (5) stylar branch fully recurved so that the stigma was in contact with the anthers. We used Welch's t-tests to compare mean curvature scores between the two species in each year. In 2005 and 2008, we also recorded whether each stigma was pollinated or not and calculated the proportion of partially recurved stigmas (i.e. those with scores of 1 - 4) that had been pollinated during that day. Additionally, in 2006 and 2007 at MRC and in 2008 for the greenhouse populations, we examined whether, as has been reported for other *Hibiscus* species, prior pollination slowed or completely prevented stylar curvature. We compared levels of style curvature between flowers that had received manual selfed pollen or manual outcrossed pollen against flowers that had been allocated to the autonomous selfing treatment. To analyse these data, we used the curvature score for each flower as the dependent variable and one-way ANOVAs with *post hoc* Tukey-Kramer comparisons.

## RESULTS

### *Floral display*

Mean corolla diameter was smaller in *H. aponeurus* ( $5.6 \pm 0.12$  cm) than in *H. flavifolius* ( $6.2 \pm 0.06$  cm;  $t_{33} = 4.9$ ,  $P < 0.0001$ ), but the length of the stylar column was longer in *H. aponeurus* ( $2.9 \pm 0.06$  cm) than in *H. flavifolius* ( $2.1 \pm 0.04$  cm;  $t_{40} = 10.4$ ,  $P < 0.0001$ ). Stylar columns were much more downwardly oriented with respect to the plane of the corolla in *H. aponeurus* ( $142.2 \pm 3.1$  degrees) than in *H. flavifolius* ( $11.7 \pm 2.3$  degrees;  $t_{55} = 8.1$ ,  $P < 0.0001$ ), giving *H. aponeurus* flowers a mildly zygomorphic shape when compared to *H. flavifolius* flowers (Fig. 1A).

### *Floral abundance and floral rewards*

Floral abundance varied across years for both species, but pooled across all surveys, we recorded approximately 6.5 times more *H. flavifolius* than *H. aponeurus* flowers (694 versus 106 respectively). The greatest difference in floral abundance was recorded in 2004 (566 *H. flavifolius* versus 77 *H. aponeurus* flowers), but strong differences remained in 2005 (53 versus 25) and 2006 (75 versus 4). Detailed comparisons between species for nectar volume, sugar concentration, and pollen scores during each time interval and year of observation are shown in supplementary information (Appendix II, Tab. S1).

**i) Nectar volume** — Relative nectar volumes in the two species varied across years of observation. In 2005, nectar levels early in the morning were similar ( $F_{1,3} = 1.7$ ,  $P = 0.280$ ; Fig. 2A), but by midday nectar volume in *H. flavifolius* was significantly lower ( $F_{1,3} = 633.6$ ,  $P = 0.00014$ ; Fig. 2A), and by the end of the day half of the flowers of this species contained no measurable nectar ( $< 0.1$   $\mu$ l). In contrast, in 2005 nectar standing crop in *H. aponeurus* flowers averaged above  $0.6$   $\mu$ l / flower in all three time intervals (Fig. 2A). In 2006, early morning standing crop nectar volume for *H. flavifolius* flowers was on average more than twice that recorded in *H. aponeurus* ( $F_{1,8} = 7.6$ ,  $P = 0.025$ ), but later in the day volumes decreased in both species and did not differ significantly (Fig. 3A).

**ii) Sugar concentration** — Nectar sugar concentration did not differ significantly between the two species in any time interval for either of the two years of observation (Figs. 2B and 3B). In *H. aponeurus*, sugar concentration ranged from 29% - 48% in 2005 and 38% - 41% in 2006, whereas in *H. flavifolius*, respective estimates were 20% - 46% and 32% - 39%.

**iii) Pollen availability** — In 2005 and 2006, pollen availability scores for *H. flavifolius* flowers declined strongly through time on all sampling days, with nearly all pollen removed by the end of the day (mean scores 0.03; Figs. 2C and 3C). In contrast, mean pollen scores for *H. aponeurus* remained above 3.0 throughout the day on all days and years of observation, indicating very low pollen removal by floral visitors. Consequently, end-of-day pollen scores for 2005 and 2006 differed significantly between species ( $P < 0.0001$ ). Differences were less pronounced in 2008, but end-of-day pollen availability was still greater in *H. aponeurus* ( $2.42 \pm$

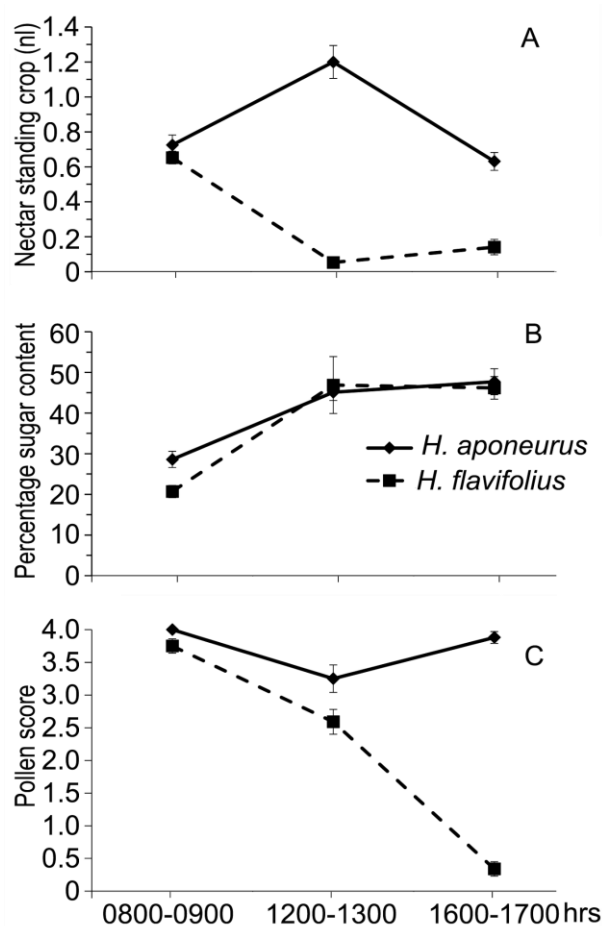


FIGURE 2. Change in floral resources for *H. aponeurus* and *H. flavifolius* as recorded in 2005 during three daily time periods at Mpala Research Centre. Graphs show A) nectar standing crop ( $\mu\text{L}$  / flower); B) nectar sugar concentration (% sugar); and C) pollen availability (as pollen score). Data points are grand means  $\pm$  S.E. across all flowers (25 - 49 per species) and sampling dates (2 days for *H. aponeurus* and 3 days for *H. flavifolius*). The percentage of pollen remaining in anthers was scored on a scale of 0 - 4 (0 = < 1%; 1 = 1 - 25%; 2 = 26 - 50%; 3 = 51 - 75%; 4 = 76 - 100%).

0.15;  $N = 36$ ) flowers than in *H. flavifolius* ( $2.00 \pm 0.09$ ;  $N = 75$ ) flowers ( $F_{1,16} = 11.0$ ;  $P = 0.001$ ).

#### Insect visitation

Across four years of observation (2004 - 2008), we recorded 245 visits to *H. aponeurus* flowers, and 663 visits to *H. flavifolius* flowers (see Appendix III, Tab. S2 for a taxonomic checklist). However, our 2008 data revealed that only a minority of the visits to either species involved insects landing in the forward-facing position that facilitates pollen transfer onto the stigmas. These potentially effective visits were significantly more frequent (Fisher's exact test,  $P = 0.001$ ) for *H. flavifolius* (21%,  $N = 107$ ) than for *H. aponeurus* (0%,  $N = 36$ ).

i) Diversity of visitor assemblages — Across all four years, the composition of visitor assemblages and the relative abundance of the visiting taxa differed significantly between *Hibiscus* species (examining four categories: bees, butterflies,

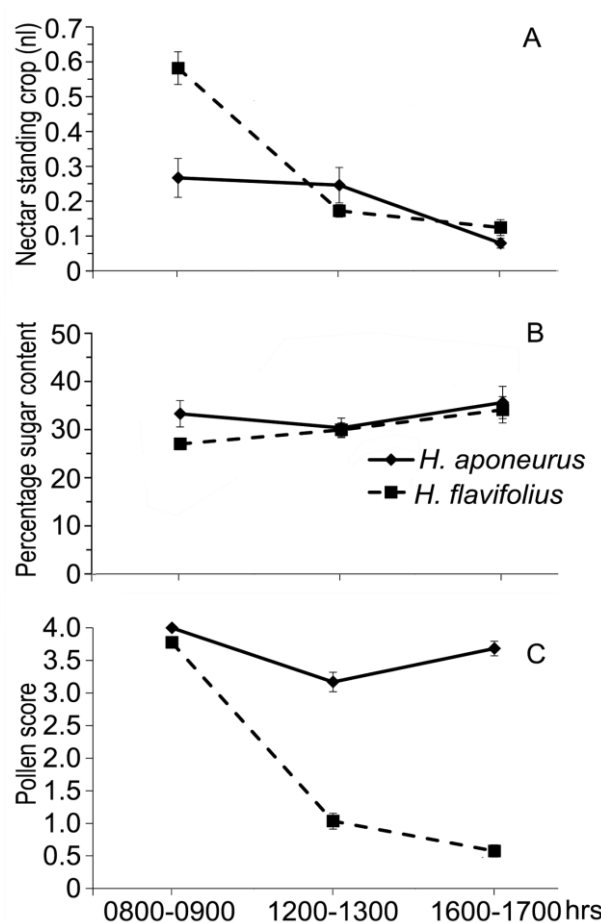


FIGURE 3. Change in floral resources for *H. aponeurus* and *H. flavifolius* as recorded in 2006 during three daily time periods at Mpala Research Centre. Graphs show A) nectar standing crop ( $\mu\text{L}$  / flower); B) nectar sugar concentration (% sugar); and C) pollen availability (as pollen score). Data points are grand means  $\pm$  S.E. across all flowers (46 - 91 per species) and sampling dates (5 days for each species). The percentage of pollen remaining in anthers was scored on a scale of 0 - 4 (0 = < 1%; 1 = 1 - 25%; 2 = 26 - 50%; 3 = 51 - 75%; 4 = 76 - 100%).

beetles, other insects:  $\chi^2 = 251.7$ ,  $df = 3$ ,  $P < 0.0001$ ; Fig. 4). In both plants, the most common visitor taxa were bees, and the rarest were bugs and flies. Five and six bee genera, respectively, visited *H. aponeurus* and *H. flavifolius*, and four of those genera (*Apis mellifera*, *Braunsapis* sp., *Lasioglossum* sp., and *Tetralonia* sp.) were recorded visiting flowers of both species (Appendix III, Tab. S2). Bee visits accounted for 87.5% of the total to *H. flavifolius* and 46.1% of the total to *H. aponeurus*. By contrast, butterflies were both more diverse and more frequently recorded visiting *H. aponeurus* flowers (nine genera; 41.2% of visits) than *H. flavifolius* flowers (three genera; 2.7% of visits). Two main beetle genera, *Coryna* and *Meloidea*, were observed consuming the flowers of both species (11.6% of visits to *H. aponeurus*; 6.3% to *H. flavifolius*), and it is possible that in a few cases they facilitated autogamy by knocking pollen onto the stigmas.



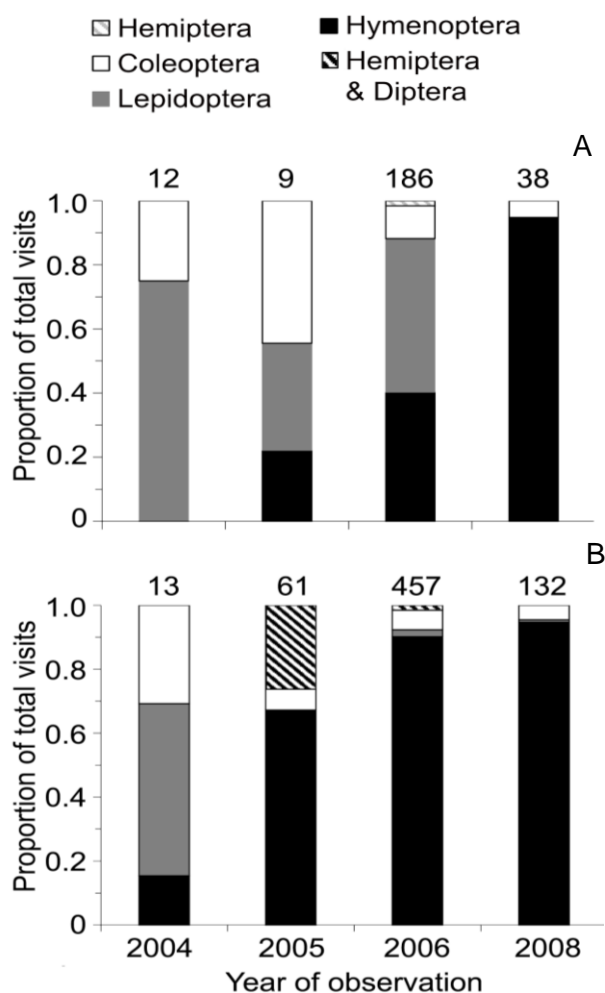


FIGURE 4. Diversity of insect taxa grouped by order that were observed visiting A) *H. aponeurus* and B) *H. flavifolius* flowers in 2004, 2005, 2006, and 2008 at Mpala Research Centre. Total numbers of recorded visits are given above each column.

Patterns of visitation differed significantly between *Hibiscus* species in 2005 (bees *versus* other insects; Fisher's

exact test,  $P = 0.022$ ) and 2006 (bees *versus* butterflies *versus* other insects;  $\chi^2 = 214.3$ ,  $df = 2$ ,  $P < 0.0001$ ). However, visitor frequencies did not differ significantly between plant species during 2004, when most visitors were butterflies, or in 2008, when over 90% of all visits were by bees (mostly honeybees, *Apis mellifera*, a species not recorded in previous years).

ii) Floral visitation rates per hour — Across all visitor taxa, *H. flavifolius* flowers received five times as many visits per hour as did *H. aponeurus* in 2005, and about twice as many in both 2006 and 2008 (Tab. 1). Visitation rates by bee visitors alone were 16 times greater to *H. flavifolius* than to *H. aponeurus* in 2005, and nearly five times greater in 2006 (Tab. 1). Across all surveys in 2008, *H. aponeurus* flowers that had received higher numbers of visits had significantly less pollen remaining in their anthers at the end of the day ( $R^2 = 0.73$ ,  $P = 0.003$ ). Although we observed a similar trend in *H. flavifolius*, this relationship was not statistically significant ( $R^2 = 0.23$ ,  $P = 0.194$ ).

#### Tests for apomixis, pollen limitation, autofertility, and reproductive assurance

None of the apomixis treatments produced fruits in either species, whether at MRC in 2005 or 2007 (54 and 11 *H. aponeurus* flowers; 66 and 6 *H. flavifolius* flowers, respectively), or in the greenhouse (26 *H. aponeurus* and 3 *H. flavifolius* flowers). Both species appear to be highly self-compatible and to experience negligible levels of early acting inbreeding depression. Fruit set from manual selfing was 61–98% at MRC (Tab. 2) and 91–92% in the greenhouse (Tab. 3). These levels did not differ significantly from fruit set from manual outcrossing, except for *H. aponeurus* in 2005 (Tab. 2), when fruit set was much higher in the manually selfed flowers than for other treatments ( $\chi^2 = 11.3$ ,  $df = 3$ ,  $P = 0.01$ ). Likewise, seed number per fruit for manually selfed flowers generally matched that of manually outcrossed flowers (Tabs. 2 and 3), except for the *H. flavifolius* crosses in 2005, when seed number differed significantly among treatments ( $F_{54} = 4.0$ ,  $P = 0.013$ ), and manually selfed flowers produced about 64% as many seeds as did manually outcrossed flowers (Tukey-Kramer test;  $P = 0.032$ ).

TABLE 1. Visitation rates (number of visits/flower/hour) to *H. flavifolius* and *H. aponeurus* in 2005, 2006, and 2008. Statistical comparisons use two-sample t-tests for 2005 and 2006 and paired t-tests for 2008.

Visitors - Year	<i>H. flavifolius</i>	<i>H. aponeurus</i>	$P t $
All insects			
2005	$0.25 \pm 0.03$	$0.05 \pm 0.02$	$t_8 = 4.2$ , $P = 0.025$
2006	$1.55 \pm 0.16$	$0.69 \pm 0.51$	$t_8 = 1.6$ , $P = 0.148$
2008	$0.32 \pm 0.04$	$0.18 \pm 0.04$	$t_8 = 4.2$ , $P = 0.003$
Bees only			
2005	$0.16 \pm 0.03$	$0.01 \pm 0.01$	$t_8 = 3.8$ , $P = 0.032$
2006	$1.39 \pm 0.14$	$0.28 \pm 0.25$	$t_8 = 3.9$ , $P = 0.004$
2008	$0.29 \pm 0.05$	$0.17 \pm 0.05$	$t_8 = 4.8$ , $P = 0.001$



TABLE 2. Fruit set (percentage of total flowers pollinated that developed a fruit) and mean  $\pm$  S.E. number of seeds / fruit for four pollination treatments from *H. aponeurus* and *H. flavifolius* populations at Mpala Research Centre. Numbers of maternal plants used for crosses in 2005, 2006, 2007, and 2013, respectively, were 32, 49, 33, and 32 in *H. aponeurus* and 31, 57, 35, and 27 in *H. flavifolius*. In 2007 only, we emasculated the open pollinated, manually outcrossed, and manually selfed flowers. Values in parentheses represent numbers of flowers pollinated (for fruit set) and numbers of fruits sampled (for seed number).

Species - Treatment	2005		2006	2007	2013	2013
	Fruit set	Seed number	Fruit set	Fruit set	Fruit set	Seed number
<i>Hibiscus aponeurus</i>						
Open pollination	79.0 (62)	22.2 $\pm$ 1.7 (13)	69.5 (131)	40.9 (22)	0.75 (36)	17.6 $\pm$ 1.3 (27)
Autonomous selfing	84.2 (57)	17.6 $\pm$ 3.2 (8)	72.4 (105)	61.1 (18)	---	---
Manual outcrossing	86.3 (51)	20.2 $\pm$ 2.8 (9)	74.8 (103)	65.6 (32)	81.6 (38)	16.9 $\pm$ 1.5 (31)
Manual selfing	98.0 (50)	19.2 $\pm$ 1.9 (10)	---	60.7 (28)	86.7 (15)	18.8 $\pm$ 2.7 (13)
<i>Hibiscus flavifolius</i>						
Open pollination	79.0 (100)	17.7 $\pm$ 1.8 (19)	70.3 (91)	76.9 (13)	85.7 (35)	18.5 $\pm$ 1.5 (30)
Autonomous selfing	75.0 (80)	22.6 $\pm$ 1.9 (11)	78.5 (79)	85.7 (14)	---	---
Manual outcrossing	84.5 (71)	23.1 $\pm$ 2.5 (18)	80.0 (95)	84.2 (19)	90.4 (21)	24.6 $\pm$ 1.2 (19)
Manual selfing	78.8 (85)	14.8 $\pm$ 1.7 (18)	---	88.2 (17)	87.5 (16)	22.8 $\pm$ 2.2 (14)

i) Pollen limitation — We found little evidence of pollen limitation in either species. The relative levels of fruit set for open pollinated flowers were 6 - 12% lower than for manually pollinated flowers in our 2005 and 2006 pollinations at MRC (Tab. 2), and those differences were never significant ( $\chi^2$  tests,  $df = 3$ ;  $P > 0.05$  in all cases). For seed number, only the *H. flavifolius* data for 2013 suggested pollen limitation ( $F_{2,60} = 4.26$ ;  $P = 0.019$ ; Tab. 3), with approximately a 30% reduction for open pollination relative to manual outcrossing (Tukey-Kramer test;  $P = 0.019$ ). When we eliminated the possibility of facilitated selfing in 2007 by emasculating the flowers, open-pollinated fruit set was reduced by 33 - 35% relative to all other treatments in *H. aponeurus* ( $\chi^2 = 3.53$ ,  $df = 3$ ,  $P = 0.32$ ; Tab. 2), but was reduced by only 9 - 13% relative to other treatments in *H. flavifolius* ( $\chi^2 = 0.71$ ,  $df = 3$ ,  $P = 0.87$ ; Tab. 2).

ii) Autofertility — Field and greenhouse pollinations produced contrasting results for autofertility. At MRC, fruit set for autonomously selfed bagged flowers was lower than but not significantly different from fruit set for manually outcrossed flowers (Tab. 2). Likewise, in 2005, seed number did not differ between autonomously selfed flowers and manually outcrossed flowers (Tab. 2). In the greenhouse, where we could eliminate the chances of accidental pollination due to bagging, autonomous selfing of unbagged *H. aponeurus* flowers produced roughly half the fruits recorded for the manually pollinated flowers ( $\chi^2 = 47.5$ ,  $df = 2$ ,  $P < 0.0001$ ; Tab. 3). An even larger difference between these two treatments was recorded for *H. flavifolius*, where autonomous selfing resulted in less than a third of fruit set produced by manually pollinated flowers ( $\chi^2 = 36.8$ ,  $df = 2$ ,  $P < 0.0001$ ; Tab. 3). Seed number in autonomously selfed fruits of *H. aponeurus* was 36% lower than in manually outcrossed and manually selfed fruits ( $F_{2,121} = 20.7$ ;  $P < 0.0001$ ; Tab. 3). Autonomously selfed fruits of *H. flavifolius* also contained fewer seeds than did fruits from manually pollinated flowers, but this difference was not significant (Welch's ANOVA;  $F_{2,18} = 1.5$ ;  $P = 0.248$ ).

TABLE 3. Fruit set (percentage of total flowers pollinated that developed a fruit) and mean  $\pm$  S.E. number of seeds/fruit for three pollination treatments from *H. aponeurus* and *H. flavifolius*, performed in the greenhouse at Indiana University South Bend. Values in parentheses represent numbers of flowers pollinated (for fruit set) and numbers of fruits sampled (for seed set).

Species - Treatment	Fruit set	Seed number
<i>Hibiscus aponeurus</i>		
Autonomous selfing	48.3 (60)	14.5 $\pm$ 1.1 (21)
Manual outcrossing	94.6 (56)	22.6 $\pm$ 0.8 (45)
Manual selfing	92.5 (67)	22.6 $\pm$ 0.7 (58)
<i>Hibiscus flavifolius</i>		
Autonomous selfing	30.0 (30)	20.0 $\pm$ 1.2 (9)
Manual outcrossing	100 (15)	23.5 $\pm$ 0.9 (14)
Manual selfing	91.3 (23)	23.6 $\pm$ 0.8 (20)

iii) Reproductive assurance — In our 2007 test for reproductive assurance, fruit set for *H. aponeurus* open-pollinated intact flowers was almost twice as great as for open-pollinated emasculated flowers (78% versus 41%), but that difference was not significant due to small sample sizes (9 intact and 22 emasculated flowers; Fisher's exact test,  $P = 0.11$ ). For *H. flavifolius*, fruit set from open-pollinated intact flowers (83%;  $N = 6$  flowers) was very similar to fruit set from open-pollinated emasculated flowers (77%;  $N = 13$  flowers).

#### Use of stylar curvature for delayed selfing

As predicted, our end-of-day surveys of open-pollinated flowers showed that *H. aponeurus* flowers displayed significantly greater stylar curvature than did *H. flavifolius* flowers in all four years of observation (Fig. 5). Average curvature for *H. aponeurus* consistently exceeded 90° to the long axis of stylar column (mean score always  $> 3.0$ ; see Fig. 1C), whereas this degree of curvature rarely occurred in *H. flavifolius*. For *H. aponeurus*, 75% of the styles in 2005 had

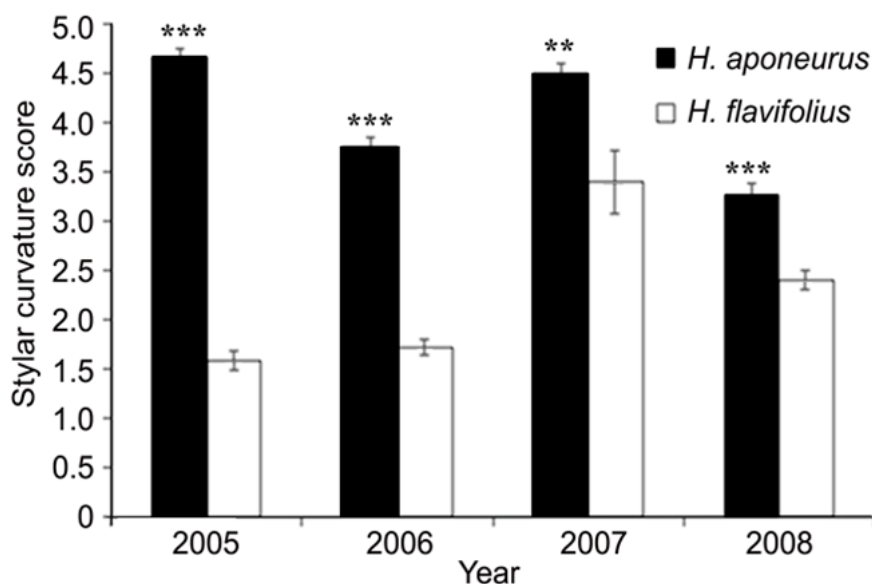


FIGURE 5. Comparison of styler curvature scores (mean  $\pm$  S.E.) for end-of-day, open-pollinated flowers of *H. aponeurus* and *H. flavifolius* at Mpala Research Centre. See Figure 1C for an illustration of curvature scores. In 2005,  $N = 27$  and 41 flowers, respectively ( $t_{60} = 22.5$ ,  $P < 0.0001$ ); in 2006,  $N = 94$  and 79 flowers ( $t_{57} = 14.7$ ,  $P < 0.0001$ ); in 2007,  $N = 40$  and 13 flowers ( $t_5 = 3.2$ ,  $P = 0.0062$ ); and in 2008,  $N = 35$  and 75 flowers ( $t_{88} = 5.8$ ,  $P < 0.0001$ ).

TABLE 4. Comparison of styler curvature scores (mean  $\pm$  S.E.) for autonomously selfed and manually pollinated flowers from 2006 and 2007 at Mpala Research Centre and for 2008 in the greenhouse at Indiana University South Bend. See Figure 1C for an illustration of curvature scores. Within years and species, treatments not sharing the same superscript are significantly different from one another (ANOVA with Tukey-Kramer tests).

Year-Pollination treatment	<i>Hibiscus aponeurus</i>		<i>Hibiscus flavifolius</i>	
	No. flowers	Curvature score	No. flowers	Curvature score
MRC 2006				
Autonomous selfing	50	4.53 $\pm$ 0.16	36	2.78 $\pm$ 0.17
Manual outcrossing	47	2.47 $\pm$ 0.17	32	1.64 $\pm$ 0.18
		$F_{1,95} = 76.7$ ; $P < 0.0001$		$F_{1,66} = 21.4$ ; $P < 0.0001$
MRC 2007				
Autonomous selfing	21	4.33 $\pm$ 0.25 <sup>A</sup>	14	3.43 $\pm$ 0.21 <sup>A</sup>
Manual selfing	37	2.75 $\pm$ 0.19 <sup>B</sup>	26	2.43 $\pm$ 0.16 <sup>B</sup>
Manual outcrossing	36	2.65 $\pm$ 0.19 <sup>B</sup>	24	2.11 $\pm$ 0.16 <sup>B</sup>
		$F_{2,91} = 16.9$ ; $P < 0.0001$		$F_{2,61} = 12.4$ ; $P < 0.0001$
IUSB greenhouse 2008				
Autonomous selfing	56	4.60 $\pm$ 0.10 <sup>A</sup>	49	4.40 $\pm$ 0.12 <sup>A</sup>
Manual selfing	66	2.48 $\pm$ 0.13 <sup>B</sup>	43	2.20 $\pm$ 0.13 <sup>B</sup>
Manual outcrossing	50	2.77 $\pm$ 0.16 <sup>B</sup>	23	2.04 $\pm$ 0.17 <sup>B</sup>
		$F_{2,169} = 76.3$ ; $P < 0.0001$		$F_{2,112} = 103.8$ ; $P < 0.0001$

completely recurved to effect self-pollination, and of those that were not fully recurved (scores 1 - 4), only 35% of the stigmas had been pollinated. In 2008, however, only 7.4% of styles were fully recurved, and the rate of pollination in the remaining styles was 67%. By contrast, *H. flavifolius* showed greater similarity between years, with low percentages of fully recurved styles (14% in 2005 and 5.1% in 2008) and high rates of pollination on stigmas with partially recurved styles (86% in 2005 and 82% in 2008).

In agreement with our prediction that pollination early in the day would delay or stop styler curvature, styles in manually pollinated flowers always curved less than styles in autonomously selfed flowers (Tab. 4). In *H. aponeurus*, whether in the field or in the greenhouse, curvature for the

autonomously selfed treatment consistently neared completion (mean scores 4.3 - 4.6, Tab. 4; Fig. 1C), and curvature for manually pollinated flowers always stopped well before the styles reached 90° (mean scores 2.5 - 2.8, Tab. 4; Fig. 1C). In *H. flavifolius*, the results for the greenhouse crosses mirrored those for *H. aponeurus*, but in the field, curvature stopped well before stigmas could contact anthers in all treatments (mean scores 1.6 - 3.4, Tab. 4).

## DISCUSSION

The sympatric co-flowering *H. aponeurus* and *H. flavifolius* offer abundant floral rewards that attract a diversity of visitor taxa, but floral visitor arrays and visitation rates strongly differ between species and across years. As a result,

much less pollen is removed and fewer stigmas are pollinated in *H. aponeurus* than in *H. flavifolius*. Despite lower levels of visitation and pollination, *H. aponeurus* appears not to be pollen limited with regard to fruit or seed set, a result that can be attributed to a combination of facilitated and autonomous selfing along with a small number of ovules that probably can be fully fertilized with deposition of only 20–30 self or outcross pollen grains. Both species clearly possess mixed mating systems that include facultative delayed autonomous selfing as a potential means of reproductive assurance, but this mechanism was observed much more frequently in *H. aponeurus* than in *H. flavifolius*. The differences in levels of delayed selfing between species correlate well with differential rates of insect visitation and the distinct floral morphology of *H. aponeurus* that appears to reduce chances of outcross pollen deposition while increasing chances for facilitated autogamy.

### Visitor assemblages and potential for pollinator competition

*Hibiscus aponeurus* and *H. flavifolius* flowers were visited by substantially different sets of insects from year to year, with *H. aponeurus* generally attracting a higher proportion of butterflies and beetles, and *H. flavifolius* attracting more bees. Although we observed no interspecific flights by bees or butterflies as we monitored visitation, competition for pollination services may still occur. Flowers of both species are open at the same time, and some visitor taxa were recorded on both species in the same year (e.g. honeybees in 2008). To corroborate such competition, we would need to analyze pollen loads and make careful observations to determine if insects contacted the stigmas of both species in ways which could facilitate heterospecific pollen transfer. Future surveys should also attempt to examine the potential role of top-down (e.g. climate) influences in insect activity patterns, which may also help explain the significant year-to-year differences in visitor assemblages recorded in our study. For example, while in 2004 a mixture of bees, butterflies, and beetles were observed visiting both *Hibiscus* species, in 2008 visitation was strongly dominated by bee taxa. Days of observation in 2008 were cooler and windier than in other years, with mean hourly temperatures in May and June of 25.8 °C and 24.2 °C, in comparison to 31.4 °C and 27.4 °C in 2004. Lower temperatures may have favoured the flight physiology of bees, which, even in small species, can maintain higher activity under cooler conditions than can other insects (Willmer & Stone 2004). Moreover, additional observations of pollen deposition in relation to floral morphology may help us determine the pollination effectiveness of certain visitor taxa. Beetles, for example, were observed to be mostly florivores, but they could have facilitated autogamy by knocking pollen onto stigmas. Even in *Hibiscus* species with strong separation between anthers and stigmas, the movements of floral visitors within a flower can sometimes knock pollen about and increase rates of deposition (Sampson et al. 2016). This seems particularly likely in *H. aponeurus* flowers that very often have stigmas located below the anthers due to the downward curving shape of the stigmatic column.

*Hibiscus aponeurus* and *H. flavifolius* flowers offer abundant pollen and similar volumes of nectar with

comparable sugar concentrations. Several lines of evidence, however, demonstrate that *H. aponeurus* receives less outcross pollen than does *H. flavifolius*. First, in 2005, 2006, and 2008, visitation rates to *H. aponeurus* were lower than to *H. flavifolius*. Except for a single unusual day in 2006, each *H. aponeurus* flower received on average no more than three visits over the course of an entire day (10-hr period of anthesis), whereas *H. flavifolius* flowers received as many as 16 visits in a day. Second, the amount of pollen remaining in anthers at the end of the day was always greater in *H. aponeurus* than in *H. flavifolius*, a pattern consistent with lower rates of pollen removal by flower visitors and a potential reduction in male function for *H. aponeurus*. The 2008 data also showed a significant negative correlation between visitation rates and the amount of pollen remaining in the anthers of *H. aponeurus* flowers, a relationship not observed in *H. flavifolius*, presumably because rates of visitation were sufficiently high or because visitors were highly efficient in harvesting pollen, so at the end of the day most flowers were nearly empty. Third, in 2005 and 2008 our end-of-day surveys showed that significantly fewer stigmas were pollinated in *H. aponeurus* than in *H. flavifolius* flowers. Fourth, data from 2008 showed that when bees landed on *H. aponeurus* flowers, all did so in a manner that minimized contact with the stigma. Fifth, *H. aponeurus* flowers often had stigmas located below the opened petal lobes, whereas *H. flavifolius* flowers always have stigmas located above the petals. The combination of a sometimes inaccessible stigma location, a downwardly curved stylar column, and the backwards landing orientation of most insect visitors in *H. aponeurus* would seem to make insect-mediated cross-pollination more difficult than in *H. flavifolius*.

Differences between the *Hibiscus* species in visitor assemblages and visitation rates could have several causes. For example, *H. flavifolius* flowers were consistently much more abundant than *H. aponeurus* flowers. As reviewed by Knight et al. (2005), smaller plant populations are expected to show lower pollinator visitation and pollen deposition than large plant populations. Bees especially tend to show floral constancy (Waser 1986; Goulson 1999; Willmer & Stone 2004), so in a particular foraging bout, we might expect them to focus on collecting nectar or pollen from *H. flavifolius*, as choosing this species over *H. aponeurus* would likely reduce flight time between flowers and result in collection of greater reward. This advantage to *H. flavifolius* might be compounded by the strong colour difference between species. The crimson red of *H. aponeurus* flowers may not be as easily seen by bees as the white *H. flavifolius* flowers, possibly because the *H. aponeurus* flowers are often set against a complex background of green foliage under acacia trees (Chittka & Waser 1997; Spaethe et al. 2001; Rodríguez-Gironés & Santamaría 2004). Thus, it is possible that *H. aponeurus* is competing for many of the same bee pollinators as *H. flavifolius*, with the outcome that the more frequently encountered, and possibly more easily detected, *H. flavifolius* receives the great majority of visits.

An alternative explanation is that despite offering similar quantities of pollen, nectar and sugar, the rewards offered by *H. aponeurus* may be perceived as having a lower nutritional value by the array of local bees. Floral scents and other

secondary metabolites may also play an important role in attracting or deterring visitors (Baker & Baker 1982; González-Teuber & Heil 2009; Byers et al. 2014). From this perspective, it is possible that different floral scents are produced by the two *Hibiscus* species, which attract different visitor taxa. Little is known about how variable floral traits are among populations of *H. aponeurus*, but studies in other species show that the chemical composition of volatiles and other floral traits can be subject to pollinator-mediated selection (see Azuma et al. 2001; Whitehead & Peakall 2009). Records from herbaria are scarce, but *H. aponeurus* has a wide geographical range that spans parts of Ethiopia, Sudan, southern Somalia, western and central Kenya, Uganda, Tanzania, Rwanda, Burundi, and the Democratic Republic of Congo (Exell 1961; Agnew & Agnew 1994; Barkhadle et al. 1994; Friis & Vollesen 1998; African Plant Database 2017). The populations at MRC thus represent some of the eastern most recorded localities for *H. aponeurus*, and it is possible that these populations arrived recently from more central parts of its geographical range, and that their floral traits may have evolved to attract pollinator guilds that are largely absent from MRC. Examining the plausibility of this explanation would require detailed observations of floral visitation and floral traits from *H. aponeurus* populations located in other areas of its geographical distribution, chemical analyses of the composition and nutritional value of its floral rewards, and feeding preference assays under controlled conditions.

#### *Pollen limitation, autofertility, and the importance of delayed selfing*

Consistent with studies of other Malvaceae (e.g. Klips & Snow 1997; Ruan et al. 2008b), both *H. aponeurus* and *H. flavifolius* are fully self-compatible. Across three years of crosses in the field and one set of greenhouse crosses, fruit set and seed number for manually selfed flowers were almost never lower than for manually outcrossed flowers. In addition, neither species showed strong or consistent evidence of pollen limitation, a result that agrees with published findings that low levels of pollen limitation are associated with intrinsic factors, such as self-compatibility and actinomorphy, and with extrinsic factors, such as high abundance, low plant diversity, and high pollinator diversity (Burd 1994; Larson & Barrett 2000; Knight et al. 2005; Vamosi et al. 2006, 2013; Alonso et al. 2010). Except for the low abundance of *H. aponeurus* at MRC and its slight tendency towards zygomorphy, our populations possess all of these traits. Moreover, both *H. aponeurus* and *H. flavifolius* have the potential to use stylar curvature as a mechanism of delayed autonomous selfing to compensate for low levels of insect-mediated pollination. However, our data are unclear on the degree to which delayed selfing provides reproductive assurance. Field crosses using intact flowers showed 79% - 100% autofertility in *H. aponeurus* and 87 - 100% autofertility in *H. flavifolius*, but in the greenhouse these rates were much lower, with only 33% autofertility in *H. aponeurus* and 27% in *H. flavifolius*. Eckert et al. (2010) pointed out that high autofertility indicates only the potential for reproductive assurance, and that in field experiments, it is often not well correlated with reproductive assurance, which can be measured as the fraction of reproduction due to autonomous selfing. Our small experiment to test directly for reproductive assurance was

ambiguous due to low sample sizes, but it showed that stylar curvature is more important in *H. aponeurus*, where 47% of fruit set could be attributed to delayed selfing, compared to only 7% that could be ascribed to delayed selfing in *H. flavifolius*.

Regardless of its effectiveness at offsetting low pollination rates, stylar curvature is clearly much more common in *H. aponeurus* flowers than in *H. flavifolius*, an event that appears to be driven by differences in insect visitation rates. In *H. aponeurus*, the extent of stylar curvature in open-pollinated flowers, which at MRC regularly received very low rates of visitation, was always similar to the curvature observed in bagged, autonomously selfing flowers, and always significantly greater than in manually pollinated flowers. In contrast, with the exception of 2007, stylar curvature for open-pollinated *H. flavifolius* flowers was similar to manually pollinated flowers, indicating that open-pollinated flowers were being pollinated at high enough rates to stop curvature. Thus, although we found both species to have approximately equal capacity to use autonomous selfing through stylar curvature as a means of reproductive assurance, low visitation rates in *H. aponeurus* appeared to force this species to resort to this mechanism more frequently than the more regularly visited *H. flavifolius*.

#### *Future directions*

Two complementary hypotheses could potentially explain the differences we found between *Hibiscus* species in the use of delayed selfing. *Hibiscus aponeurus* populations at MRC could be locally adapted to a largely selfing reproductive strategy or they could more simply be showing a phenotypically plastic response to low visitation levels by inadequate pollinators. Under either hypothesis, we expect that open-pollinated progeny tests using genetic markers (perhaps in artificial populations in the field where floral densities could be controlled) would find higher rates of selfing in *H. aponeurus* than in *H. flavifolius*. If the local adaptation hypothesis were true, then comparisons of selfed and outcrossed progeny should find less inbreeding depression in *H. aponeurus* than in *H. flavifolius*. Conditions within *H. aponeurus* populations, in fact appear to be favourable to the evolution of increased selfing: small population sizes, low levels of insect visitation, and high variability in pollinator composition across seasons (Morgan et al. 2005; Morgan & Wilson 2005; Knight et al. 2005). Consistent with this hypothesis are also the reduced size of the corolla in *H. aponeurus* compared to *H. flavifolius* and the placement of stigmas in a position that appears to decrease chances for outcrossing and to facilitate autogamy.

Additional evidence that MRC populations of *H. aponeurus* are evolving towards increased selfing could come from comparisons with populations situated in other parts of this species' geographical range. Klips & Snow (1997), for example, showed that in the United States, northern populations of *Hibiscus laevis* are capable of selfing, whereas southern populations are not, because styles in the southern populations were too long to allow stigmas to touch anthers even when fully recurved. They argued that the southern populations may experience more reliable bee pollination than the northern populations, resulting in the evolution of selfing only in the northern populations. Also, the "abundant centre"

model predicts that population sizes and densities will be greatest near the central region of a species' range and will decline towards the peripheries of the range (Brown 1984). A possible consequence of low population size and density is lower levels of outcrossing and selection for traits that increase autogamous self-fertilization (Jain 1976; Herlihy & Eckert 2005). As discussed above, because *H. aponeurus* populations at MRC represent the eastern periphery of the range, an analysis of more central populations could reveal evidence of higher pollination rates and less reliance on delayed selfing for seed production. Our future work contemplates visiting populations of these two species in other areas across their geographical ranges to perform floral visitation observations and seed collections. Because some of the differences in visitation observed between the two species may be due to differences in nectar and pollen composition, we are currently performing chemical analyses to examine sugar, amino acid, and potentially secondary metabolite contents. We are also assessing whether floral volatiles are released during anthesis or dehiscence that may help explaining attraction of floral visitors. To examine potential genetic consequences of autonomous selfing, we are developing genetic markers, which we will use to measure outcrossing levels of maternal families collected in the wild.

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## APPENDICES

Additional supporting information may be found in the online version of this article:

APPENDIX I. Information about seed germination and growth in greenhouses.

APPENDIX II. Significance level of the differences in resource production between species across time intervals and years of observation.

APPENDIX III. Floral visitors recorded for each plant species and each year of observation.

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